Daily or Intermittent Calcitriol Administration during Growth Hormone Therapy in Rats with Renal Failure and Advanced Secondary Hyperparathyroidism

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Growth hormone (GH) improves growth in children with chronic renal failure. The response to GH may be affected by the degree of secondary hyperparathyroidism and concurrent treatment with vitamin D. Forty-six rats underwent 5/6 nephrectomy (Nx) and were given a high-phosphorus diet (Nx-Phos) to induce advanced secondary hyperparathyroidism and divided into the following groups: (*1*) Nx-Phos ($n = 10$) received saline, (*2*) GH at 10 IU/kg per d (Nx-Phos+GH; $n = 9$), (*3*) GH and daily calcitriol (D) at 50 ng/kg per d (Nx-Phos+GH+daily D; $n = 8$), (*4*) GH and intermittent D (three times weekly) at 350 ng/kg per wk (Nx-Phos+GH+int D; $n = 9$), and (*5*) intact-control ($n = 10$). Serum parathyroid hormone (PTH) levels were elevated in Nx-Phos, but IGF-I levels did not change with growth hormone. Body length, tibial length, and growth plate width did not increase with either GH or calcitriol. Proliferating cell nuclear antigen staining, PTH/PTHrP receptor, bone morphogenetic protein-7, and fibroblast growth factor receptor-3 expression increased with GH alone or with intermittent calcitriol but were slightly diminished during daily calcitriol administration. GH enhanced IGF-I, IGF binding receptor-3, and GH receptor but declined with daily and intermittent calcitriol. Overall, there was no improvement in body length, tibial length, and growth plate width at the end of GH therapy, but selected markers of chondrocyte proliferation and chondrocyte differentiation increased, although these changes were attenuated by calcitriol. The combination of GH and calcitriol that is frequently used in children with renal failure and secondary hyperparathyroidism require further studies to evaluate the optimal dose and frequency of administration to increase linear growth and prevent bone disease.

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Growth hormone (GH) is a potent mitogenic agent that is frequently used to increase linear growth in children with chronic renal failure. The growth response, however, remains suboptimal in children who are maintained on chronic dialysis therapy compared with predialysis patients who are on conservative medical treatment (1). Causative factors that may contribute to the poor response to GH in dialysis children include a greater degree of GH insensitivity, increased severity of secondary hyperparathyroidism, differences in skeletal histology, concurrent treatment with vitamin D, and high doses of calcium salts.

GH stimulates proliferation in various types of cells, including chondrocytes and osteoblasts, and increases collagen production either directly by binding to the GH receptor (GHR) or indirectly by increasing hepatic and local IGF-I production (2–4). Calcitriol (1,25-dihydroxyvitamin D_3) is used on a regular basis to maintain normocalcemia, control the development and progression of secondary hyperparathyroidism, and prevent renal bone disease in pediatric patients with chronic renal failure. Studies have shown that calcitriol exerts a dose-dependent antiproliferative effect on chondrocytes and osteoblasts (5). Mehls *et al.* (6) demonstrated that daily calcitriol and not intermittent calcitriol administration increased growth rate after 2 wk of treatment in nephrectomized rats with mild secondary hyperparathyroidism when given alone or in combination with GH. In contrast, several investigators have reported that parathyroid hormone (PTH) suppression and decline in PTH levels are comparable in children with chronic renal failure who were treated with 8 wk of daily or intermittent calcitriol (7,8)

The optimal mode of calcitriol administration to control secondary hyperparathyroidism and prevent growth impairment in children with chronic renal failure remains to be determined. Reduction in linear growth and development of adynamic bone have been described in dialysis children with severe secondary hyperparathyroidism after 12 mo of high-dose intermittent calcitriol therapy (9,10). Concurrent calcitriol and GH therapy is a common practice in young children with chronic renal failure to augment linear growth and prevent bone disease. There is limited information on the effects of daily or intermittent calcitriol administration when given with GH in the presence of advanced secondary hyperparathyroidism. Thus, the objective of the current study was to evaluate bone growth, selected markers of chondrocyte proliferation, and chondrocyte maturation in the growth plate of young rats that had advanced secondary hyperparathyroidism and were treated with GH and daily or intermittent calcitriol.

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Materials and Methods

Forty-six male weanling Sprague-Dawley rats that weighed 62 ± 3 g (Harlan Laboratories, Madison, WI) were housed in individual cages and given standard rodent diet (23.4% protein, 0.6% calcium, 0.6% phosphorus; Purina Mills, Indianapolis, IN). After 24 h of acclimatization, 36 animals underwent the two-stage 5/6 nephrectomy as described previously (11). For inducing advanced secondary hyperparathyroidism, all nephrectomized animals were fed a high-phosphorus diet (Nx-Phos; 1.2% phosphorus; Purina Mills). Marrow fibrosis has been previously described in our phosphorus-loaded rats 4 wk after the second-stage nephrectomy (11). Ten rats underwent sham nephrectomy (intact-control) in two stages corresponding to the time of the two-stage 5/6 nephrectomy. All sham-nephrectomized animals were fed the standard rodent diet. All procedures were reviewed and approved by the Research Animal Resource Center at the University of Wisconsin (Madison, WI).

Body weight and body length measurements were obtained weekly. For ensuring equivalent caloric intake, the intact-control animals were provided the amount of food consumed by the nephrectomized rats the previous day. Six days after the second-stage surgery, nine rats received GH (Genentech, San Francisco, CA) at a dose of 10 IU/kg per d (Nx-Phos+GH), eight animals received GH at a dose of 10 IU/kg per d and daily calcitriol at a dose of 50 ng/kg per d (Nx-Phos+GH+daily D), and nine rats were given GH at a dose of 10 IU/kg per d and intermittent calcitriol at a dose of 50 ng/kg per d or 350 ng/kg per wk administered three times per week (Nx-Phos+GH+int D). Ten nephrectomized animals (Nx-Phos) and 10 sham-nephrectomized rats (intact-control) received saline injections. All injections were administered by intraperitoneal route at the same time of the day for a total of 3 wk. The dose of GH and calcitriol therapy was based on an earlier study that showed changes in the growth plate only after 10 d of treatment (11).

At the end of the 4-wk study period, the rats were killed and underwent transcardiac perfusion with 4% paraformaldehyde in PBS. Blood was obtained for serum calcium, creatinine, phosphorus, urea nitrogen, PTH, and IGF-I. The proximal tibiae were excised, and tibial length measurements were obtained. Bones were decalcified in 15% EDTA in PBS and embedded in paraffin. Sections of bone were obtained for morphometric analysis and immunohistochemistry studies.

Serum Biochemical Determinations

Serum was obtained by centrifugation, and samples were stored at −70°C. Serum urea nitrogen, creatinine, calcium, and phosphorus levels were measured using standard laboratory methods. PTH levels were measured using the Rat BioActive PTH ELISA assay kit (Immutopics, San Clemente, CA), and IGF-I levels were measured using the Rat IGF-I ELISA assay kit (Diagnostic Systems Laboratories, Webster, TX). The BioActive PTH assay detects the full-length biologically active form of rat PTH; the antibody that recognizes the C-terminal fragments are biotinylated for capture, and the other antibody that recognizes the initial N-terminal epitope is conjugated with horseradish peroxidase for detection. Blood was collected 48 h after the last dose of intermittent calcitriol and 24 h after the last dose in rats that received daily calcitriol.

Growth Plate Morphometry

For morphometric analysis, three 5-μm sections of bone were obtained, stained with hematoxylin and eosin, and viewed by light microscopy at ×30, and images were captured onto a computer monitor. The total width of the growth plate at the proximal end of each tibia was measured at equally spaced intervals using an image analysis software (Kontron 200, Kontron Instruments Ltd, Hallbergmoos, Germany) (11). The widths of the zones occupied by hypertrophic chondrocytes and proliferative chondrocytes were also measured by the same method.

Immunohistochemistry Experiments

Immunohistochemistry experiments were performed using methods described previously (12) with the following primary antibodies: IGF-I (Upstate Biotechnology, Lake Placid, NY) at 10 μg/ml, IGF binding protein-3 (IGFBP-3) at 4 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA), GHR at 20 μg/ml (American Diagnostica, Stamford, CT), PTH/PTHrP receptor at 4.4 μg/ml (Upstate Biotechnology), bone morphogenetic protein (BMP-7) at 5 μg/ml (Santa Cruz Biotechnology), and fibroblast growth factor receptor-3 at 5 μg/ml (Santa Cruz Biotechnology). Briefly, the specimens were incubated with the primary antibody at 4°C overnight in a humidified chamber. The slides then were incubated with the secondary antibody, extravidin peroxidase, developed with diaminobenzidine and counterstained with 1% methylgreen. For proliferating cell nuclear antigen (PCNA) staining, the tissues were immunostained with the mouse anti-PCNA antibody (Zymed Laboratories, South San Francisco, CA) using the manufacturer's protocol. For quantification of the protein expression, the number of cells expressing IGF-I, IGFBP-3, GHR, PTH/PTHrP receptor, BMP-7, FGFR-3, and PCNA were counted and expressed as percentage of the labeled cells over the total number of cells in the appropriate zone in the growth plate (labeling index).

Statistical Analyses

All results are expressed as mean values ± 1 SD. Data were evaluated by one-way ANOVA, and comparisons among groups were done using Bonferroni/Dunn *post hoc* tests using the StatView statistical software (SAS Institute, Cary, NC). The Pearson product moment correlation coefficient was performed to evaluate the relationship between two numerical variables. For all statistical tests, probability values <5% were considered to be significant.

Results

Serum Biochemical Parameters

Serum PTH levels increased in all nephrectomized animals by >4000% compared with the intact-control group (Table 1). Although not statistically significant, GH increased serum PTH by approximately 38% when compared with the Nx-Phos group (Table 1). Serum IGF-I levels did not increase with GH; however, there was a 60% increase in serum IGF-I levels in the rats that received GH and intermittent calcitriol (Table 1). There was no correlation between serum IGF-I and intact PTH levels ($R = -0.23$, NS); however, there was a negative correlation between serum PTH levels and tibial length measurements in nephrectomized rats ($R = -0.68$, $P < 0.001$; serum PTH levels and growth plate width in uremic rats, $R = -0.6$, $P < 0.001$).

Serum calcium levels were much lower in the Nx-Phos group and increased with both GH and calcitriol similar to intact-control; serum phosphorus, serum creatinine, and urea nitrogen levels were elevated in all nephrectomized animals (Table 1).

Anthromorphic Measurements

Weight gain did not differ between nephrectomized and control animals (Table 2). Body length was approximately 20 to 30% shorter and tibial length measurements were 10% smaller in the Nx-Phos animals compared with the intact-control

Table 1. Serum biochemical parameters in all groups obtained at the end of the study period

Serum Levels	Nx-Phos ($n = 10$)	Nx-GH ($n = 9$)	Nx-Phos+GH +Daily D ($n = 8$)	Nx-Phos+GH +Int D ($n = 9$)	Intact-Control ($n = 10$)
PTH[a] (pg/ml)	1035 ± 420[b]	1435 ± 418[b]	1289 ± 428[b]	1270 ± 616[b]	26 ± 5
IGF-I (ng/ml)	544 ± 135	599 ± 234	687 ± 309	974 ± 425	475 ± 139
Calcium (mg/dl)	7.7 ± 1.3[c]	8.6 ± 0.9	9.5 ± 1.3	8.5 ± 1.3	9.5 ± 0.2
Phosphorus (mg/dl)	14 ± 4[b]	15 ± 4[b]	16 ± 3[b]	15 ± 4[b]	10 ± 1
Urea nitrogen (mg/dl)	70 ± 23[b]	91 ± 35[b]	85 ± 28[b]	89 ± 28[b]	20 ± 3
Creatinine (mg/dl)	0.8 ± 0.1[b]	1.0 ± 0.3[b]	1.1 ± 0.4[b]	1.1 ± 0.3[b]	0.3 ± 0.1

[a]PTH, parathyroid hormone.
[b]$P < 0.006$ *versus* intact-control group.
[c]$P < 0.01$ *versus* all groups.

Table 2. Body weight, body length, and tibial length measurements in all groups

	Nx-Phos ($n = 10$)	Nx-Phos+GH ($n = 9$)	Nx-Phos+GH+ Daily D ($n = 8$)	Nx-Phos+ GH+Int D ($n = 9$)	Intact-Control ($n = 10$)
Weight gain (g)	122 ± 33	119 ± 35	115 ± 32	105 ± 30	129 ± 22
Length gain (cm)	10 ± 3.0[a]	11 ± 2.0[a]	11 ± 2.0[a]	11 ± 2.0[a]	13 ± 1.1
Tibial length (cm)	3.4 ± 0.3[a]	3.5 ± 0.2[a]	3.5 ± 0.1[a]	3.5 ± 0.1[a]	3.7 ± 0.1

[a]$P < 0.01$ *versus* intact-control group.

group, and there was no significant improvement with either GH or calcitriol after 3 wk of treatment (Table 2).

Growth Plate Morphometry

The total width of the growth plate cartilage was 20% narrower in the phosphorus-loaded rats compared with the intact-control group (Figure 1). Three weeks of GH therapy alone or combined with calcitriol did not increase the width of the growth plate (Figure 1). The ratio of the width of the proliferative zone in relation to the total growth plate was much smaller in the Nx-Phos animals compared with the intact-control group (0.5 ± 0.01 *versus* 0.6 ± 0.04; $P < 0.008$). GH and calcitriol increased the width of the proliferative zone comparable to the intact-control group. There were no differences in the ratio of the width of the hypertrophic zone to the total growth plate in all groups (0.4 ± 0.02; NS). The growth plate architecture was disorganized in the phosphorus-loaded animals and did not improve with GH or calcitriol treatment (Figure 1).

To assess chondrocyte proliferation, we evaluated PCNA staining in all groups. PCNA was 180-fold lower in the Nx-Phos group compared with intact-control and GH alone or combined with intermittent calcitriol upregulated PCNA expression in proliferating chondrocytes similar to animals with normal renal function (Figure 2). Daily calcitriol diminished the effects of GH on PCNA staining by almost 47% (Figure 2). There was a significant correlation between PCNA staining and tibial length measurements ($R = 0.4$, $P < 0.05$) and between PCNA expression and IGF-I staining ($R = 0.5$, $P < 0.005$).

PTH/PTHrP receptor and Indian hedgehog play important roles in the regulation of chondrocyte differentiation in the growth plate cartilage. In agreement with our previous experiments, PTH/PTHrP receptor protein expression declined by approximately 40% in the Nx-Phos group compared with the intact-control group (Figure 3). Treatment with GH alone or with intermittent calcitriol increased PTH/PTHrP receptor protein expression by >40-fold, comparable to the intact-control group (Figure 3). In contrast, treatment with daily calcitriol and GH increased PTH/PTHrP receptor expression by only 20% (Figure 3). PTH/PTHrP receptor protein expression correlated with PCNA staining ($R = 0.7$, $P < 0.001$), but there was no correlation demonstrated between PTH/PTHrP receptor and tibial length measurements ($R = -0.007$, NS) and between PTH/PTHrP receptor and serum intact PTH levels ($R = -0.01$, NS).

IGF-I, IGFBP-3, and GHR protein expression were localized to the lower proliferative and hypertrophic chondrocytes in the growth plate cartilage (Figures 4 through 6). As reported in our previous experiments (11), IGF-I protein and IGFBP-3 staining declined by >40% in the Nx-Phos animals compared with animals with normal renal function (Figures 4 and 5). GH increased IGF-I, IGFBP-3, and GHR staining equivalent to the intact-control group (Figure 4 through 6); however, calcitriol, whether given daily or intermittently, attenuated these effects (Figure 4 through 6). IGF-I protein expression significantly correlated with PCNA staining ($R = 0.5$, $P < 0.005$), with PTH/PTHrP receptor ($R = 0.6$, $P < 0.001$), with GHR expression ($R = 0.7$, $P < 0.001$), and with IGF binding receptor 3 ($R = 0.7$, $P < 0.001$). There was no

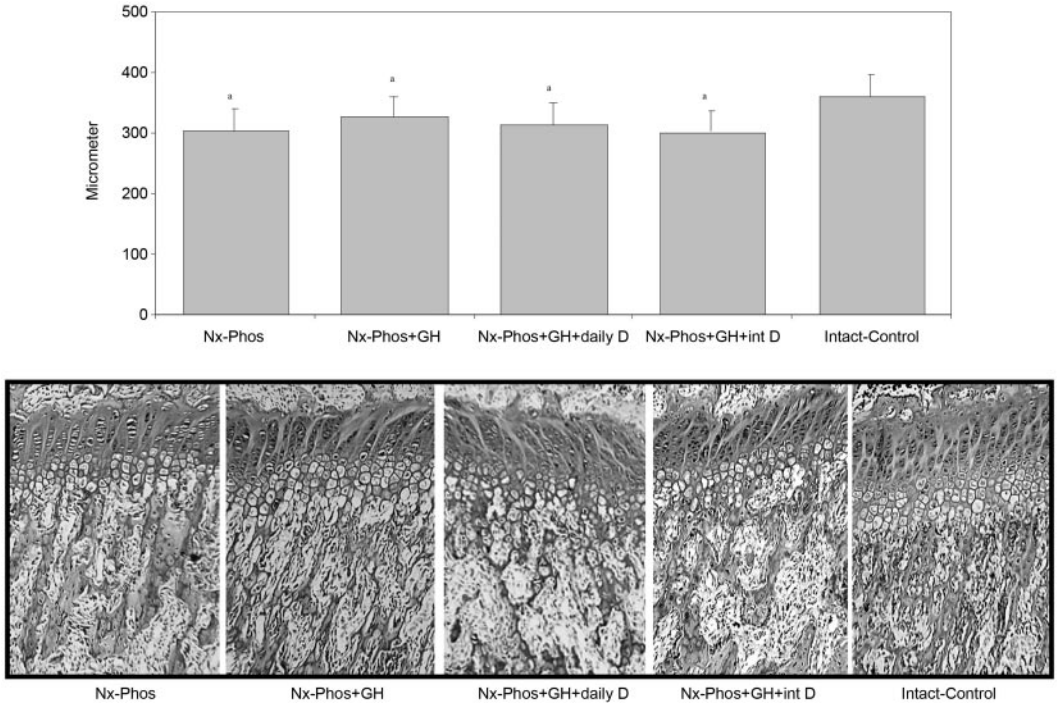

Figure 1. Top, Growth plate width measurements in all groups; $^{a}P < 0.03$ *versus* intact-control group. Bottom, Photomicrograph in all experimental groups. Note the disorganized growth plate architecture in the growth plate of phosphorus-loaded animals compared with the intact-control group. Magnification, ×30 in bottom.

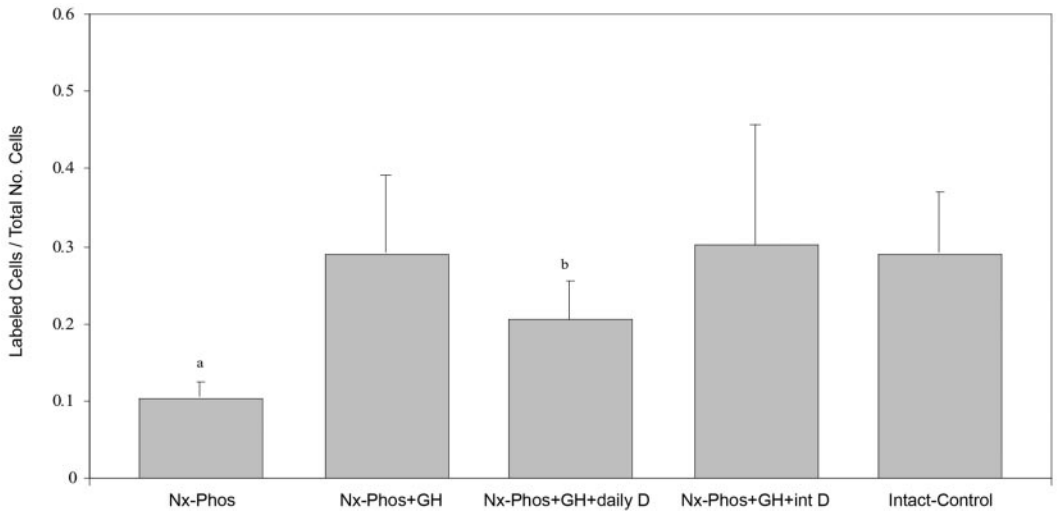

Figure 2. Quantification of the proliferating cell nuclear antigen staining expressed in the proliferating chondrocytes compared with the total number of cells in the same zone (labeling index); $^{a}P < 0.004$ *versus* all groups, $^{b}P < 0.05$ *versus* Nx-Phos+GH, Nx-Phos+GH+int D, and intact-control groups.

correlation between IGF-I protein expression and growth plate width, tibial length, gain in length, or serum IGF-I levels obtained at the end of the study period.

Chondrocyte maturation was evaluated by the expression of BMP-7 and fibroblast growth factor receptor-3 (FGFR-3). BMP-7 and FGFR-3 staining expression were much less in the Nx-Phos animals compared with the intact-control group (Figures 7 and 8). BMP-7 and FGFR-3 protein staining increased by approximately 45-fold during GH treatment alone or combined with intermittent calcitriol, similar to the intact-control group (Figures 7 and 8). In contrast, daily calcitriol lessened the effects of GH on BMP-7 and FGFR-3 protein expression, similar to the Nx-Phos group (Figures 7 and 8). BMP-7 protein expression correlated with PTH/PTHrP receptor ($R = 0.7$, $P < 0.001$), with IGF-I protein ($R = 0.7$, $P < 0.001$), and with FGFR-3 ($R = 0.7$, $P < 0.001$).

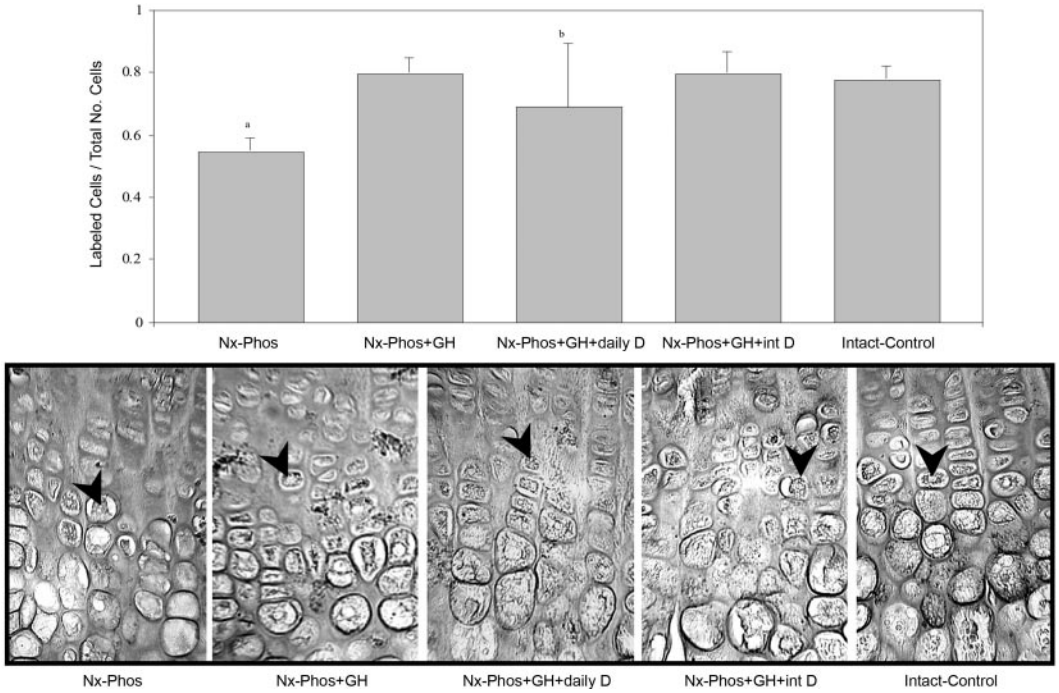

Figure 3. Parathyroid hormone (PTH)/PTHrP receptor protein expression in the growth plate of all groups. Top, Quantification of the protein expression. Bottom, Positive staining denoted by the arrows; $^{a}P < 0.0001$ *versus* all groups; $^{b}P < 0.01$ *versus* Nx-Phos+GH, Nx-Phos+GH+int D, and intact-control groups. Magnification, ×50 in bottom.

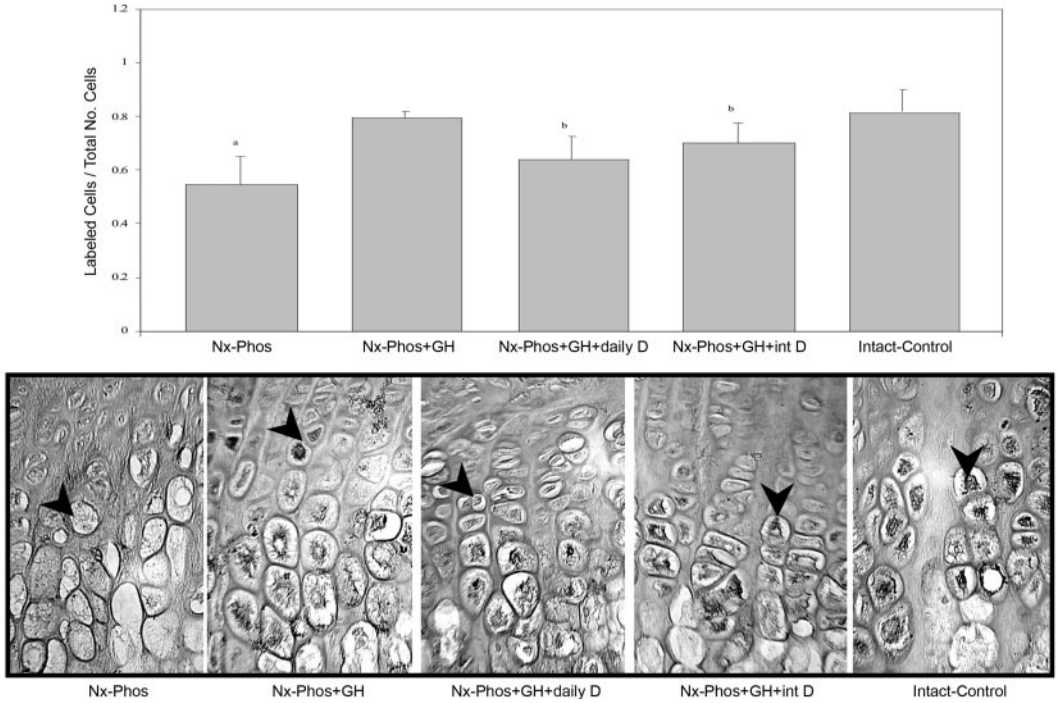

Figure 4. Top, Quantification of the response to growth hormone (GH) and calcitriol. Bottom, IGF-I protein expression in the lower proliferative and in the hypertrophic chondrocytes, denoted by arrows. $^{a}P < 0.0001$ *versus* all groups; $^{b}P < 0.01$ *versus* Nx-Phos+GH and intact-control groups. Magnification, ×50 in bottom.

Discussion

The results of the current study suggest that GH alone or in combination with calcitriol may not improve body length or increase bone growth in rats with renal failure and advanced secondary hyperparathyroidism; these results may concur with the reported suboptimal growth response in children who are

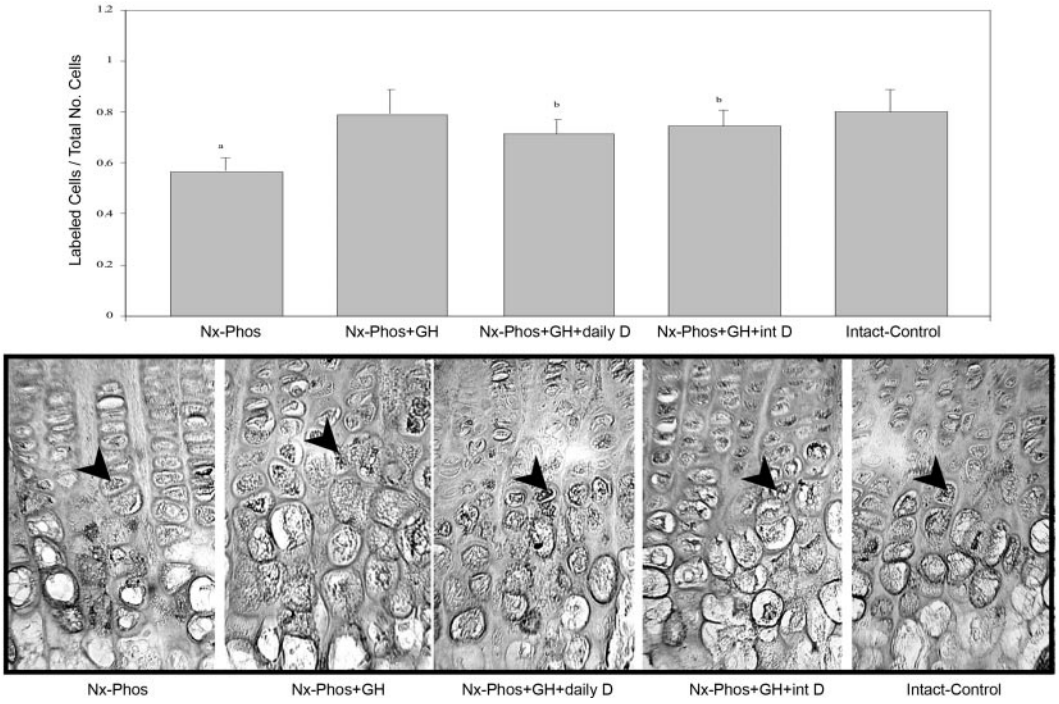

Figure 5. IGF binding protein-3 protein expression in the growth plate cartilage. Top, Quantification of the protein expression (labeling index). Bottom, Positive staining denoted by dark staining and arrows. [a]$P < 0.0001$ *versus* all groups; [b]$P < 0.04$ *versus* Nx-Phos+GH and intact-control groups. Magnification, ×50 in bottom.

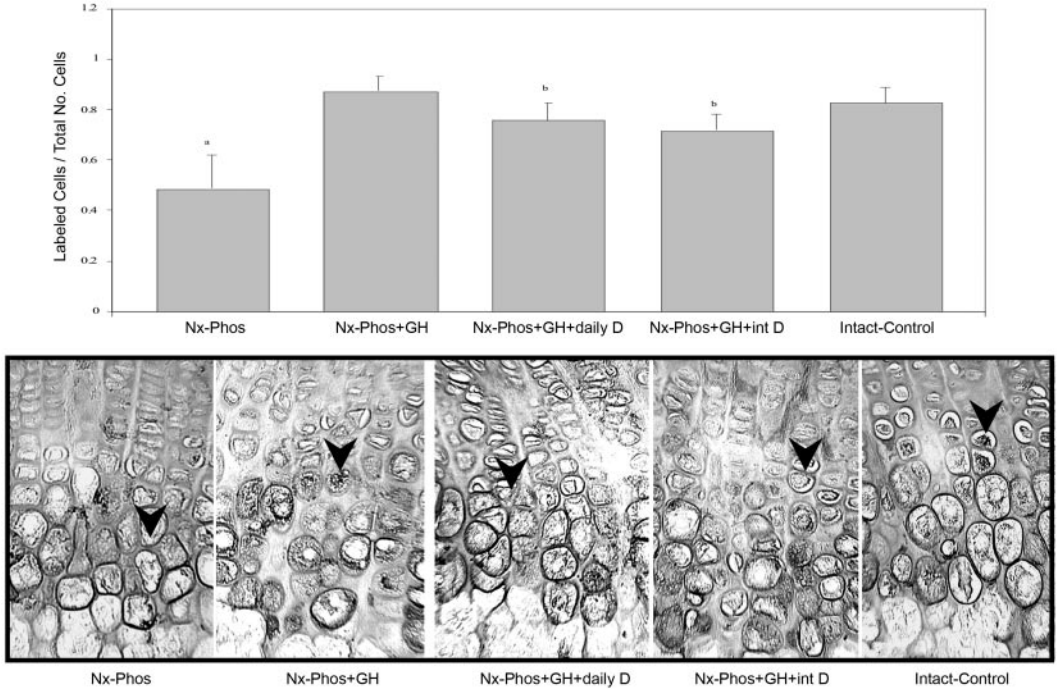

Figure 6. Top, Quantification of the response to GH and calcitriol. Bottom, GH receptor protein expression, denoted by the arrows. [a]$P < 0.0001$ *versus* all groups, [b]$P < 0.01$ *versus* Nx-Phos+GH and intact-control groups. Magnification, ×50 in bottom.

maintained on dialysis. Although 3 wk of treatment with GH increased selected markers of chondrocyte proliferation and chondrocyte maturation, concurrent calcitriol administration attenuated these effects on the growth plate cartilage. Whether given daily or three times weekly, calcitriol decreased GH-induced increases in IGF-I, IGFBP-3, and GHR protein expression.

We showed previously that 10 d of GH increased growth

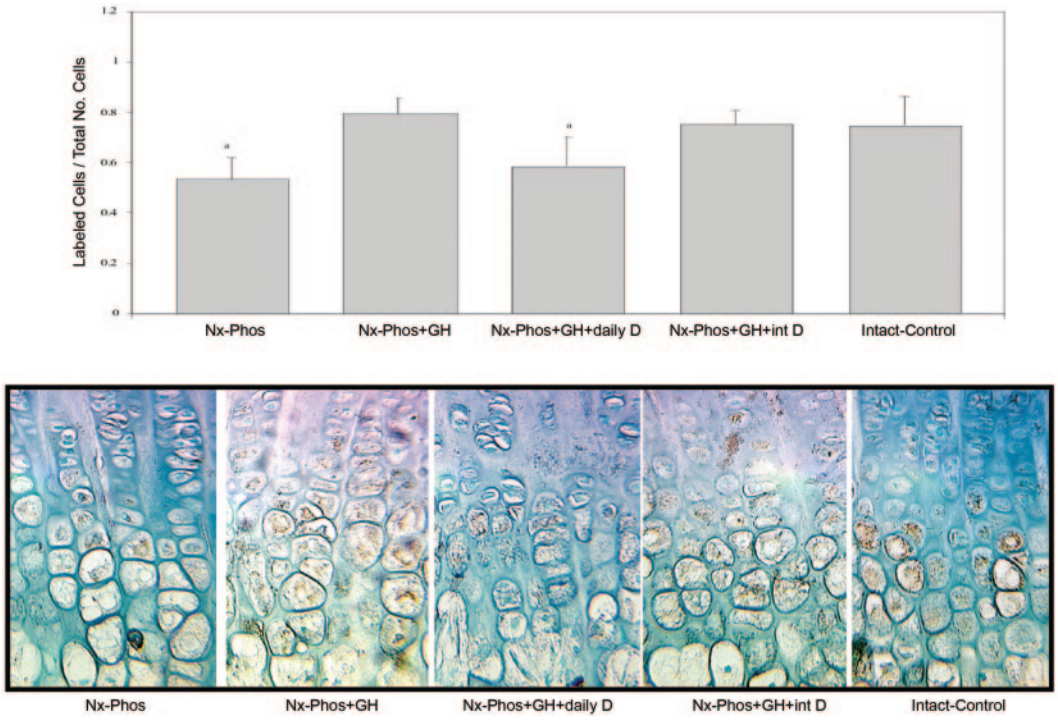

Figure 7. Top, Quantification of the response. Bottom, BMP-7 or OP-1 protein expression mostly localized to the hypertrophic chondrocytes, denoted by brown color. $^{a}P < 0.0001$ *versus* Nx-Phos+GH, Nx-Phos+GH+int D, and intact-control groups. Magnification, ×30 in bottom.

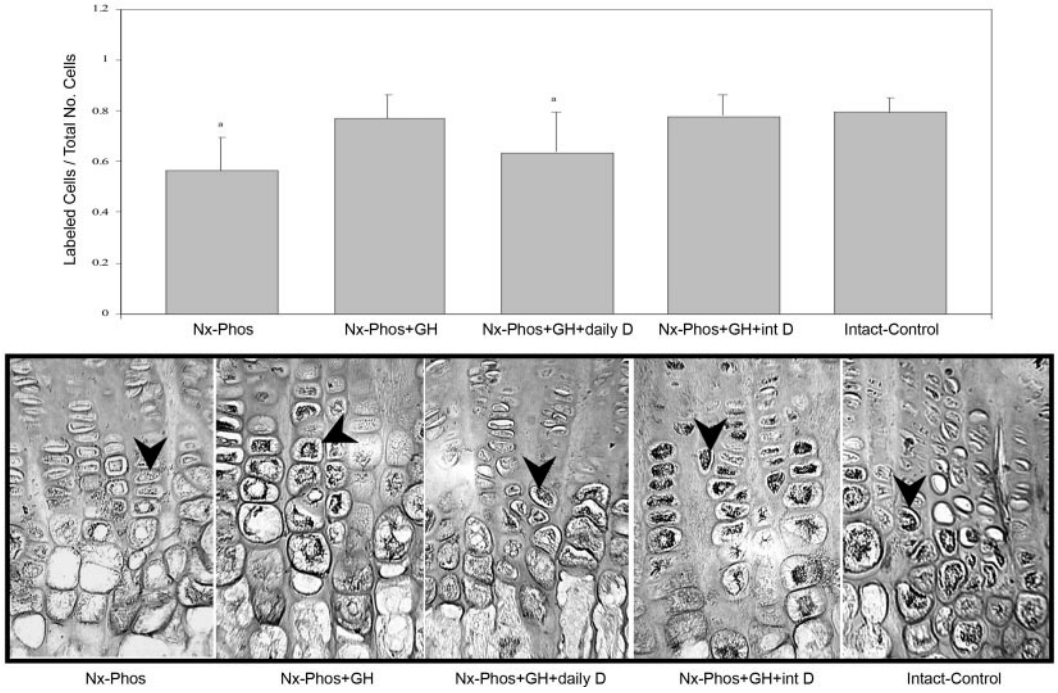

Figure 8. Top, Quantification of the response. Bottom, FGFR-3 receptor protein expression localized to the hypertrophic chondrocytes, denoted by arrows. $^{a}P < 0.0001$ *versus* Nx-Phos+GH, Nx-Phos+GH+int D, and intact-control groups. Magnification, ×30 in bottom.

plate width and when given in combination with calcitriol decreased serum PTH levels in rats with renal failure and advanced secondary hyperparathyroidism (11). In the current experiments, however, serum PTH levels that were measured using the BioActive assay are considerably much higher (557 ± 157 *versus* 1435 ± 418 pg/ml) and may reflect a greater degree

of GH insensitivity, leading to greater attenuation of GH effects and greater resistance to calcitriol therapy.

The PTH assay used in the current study measures the biologically active N-terminal PTH epitope, and we were unable to quantify the 7-84 PTH fragment because of the limited amount of serum available for each animal. Slatopolsky *et al.* (13) reported that the calcemic and phosphaturic response to PTH was attenuated by the concurrent administration of the biologically inactive PTH fragments (7-84). The presence of these inactive PTH fragments may contribute to the greater degree of insensitivity to GH therapy. The higher serum PTH levels in the current study may have diminished the anabolic effects of GH, because we showed previously that serum intact PTH levels >500 pg/ml were already associated with moderate to severe marrow fibrosis in phosphorus-loaded nephrectomized rats (11).

In the current study, calcitriol given three times a week may have selectively enhanced BMP-7, FGFR-3, PCNA, and PTH/PTHrP receptor staining compared with daily calcitriol. Such findings may be due in part to the lower serum levels of calcitriol at the end of intermittent administration compared with daily treatment, 48 *versus* 24 h. Mehls *et al.* (6) demonstrated that the mean nadir serum iPTH levels obtained after 24 h were comparable in nephrectomized rats that received calcitriol either by continuous infusion or by bolus administration. Although the serum PTH levels obtained at the end of the study period were equivalent in all groups that received both GH and calcitriol, the intermittent administration of calcitriol may be associated with oscillations of PTH levels in the nephrectomized rats. Schmitt *et al.* (14) also reported that intermittent PTH administration improves body growth and bone mineral density in uremic rats. These results may indicate that fluctuations in serum PTH induced by calcitriol, exogenous PTH, or administration of calcimimetic agents may exert an anabolic effect on bones in animals with renal failure. We did not evaluate bone mass measurements in the current study, but the increase in the selected markers of chondrocyte maturation (*e.g.*, BMP-7) may suggest improvement in mineralization in these animals.

The actions of GH are transduced by direct activation of the tyrosine kinase Janus kinase 2 (JAK_2) and phosphorylation of the STAT proteins, particularly $STAT_5$ (2). The activation of the GHR also initiates the activation of the negative regulatory pathways to terminate GH signaling, including suppressors of cytokine signaling (SOCS) and cytokine inducible SH_2-containing proteins (CIS) (2). Pretreatment of UMR-106 osteoblast-like cells with calcitriol prolonged GH signaling through the inhibitory effects of calcitriol on the expression of SOCS, CIS, and other negative regulatory pathways that terminate GH signaling (15). In the presence of renal failure, however, Schaefer *et al.* (16) described a postreceptor defect in GH transduction characterized by impairment of phosphorylation and nuclear translocation STAT proteins mediated at least in part by overexpression of SOCS protein. The attenuation of the response to GH therapy in renal failure may also be partly due to an increase in the expression of the inhibitory protein SOCS (secondary to the renal failure *per se*) and the inability of calcitriol in the presence of advanced secondary hyperparathyroidism to prevent the inhibitory actions of SOCS and CIS, leading to early termination of GH signaling.

IGF-I, GHR, and IGFBP-3 expression in the growth plate all were downregulated when GH was combined with calcitriol. Although there was an increase in BMP-7 and FGFR-3 staining during intermittent calcitriol therapy, there was no improvement in body length and tibial growth when administered in the presence of advanced secondary hyperparathyroidism. When given in higher doses, calcitriol attenuated the effects of IGF-I on DNA synthesis, colony formation, and cell proliferation in rat epiphyseal chondrocytes (17). It is interesting to note that PCNA staining correlated with tibial length measurements, IGF-I protein, and PTH/PTHrP receptor staining in the current study. The number of proliferating chondrocytes may in part predict bone growth in rats with renal failure and advanced secondary hyperparathyroidism.

Calcitriol was demonstrated in our earlier studies to attenuate the mitogenic effects of GH in the growth plate of rats with renal failure (11). Calcitriol has been demonstrated to inhibit cell proliferation in various cells, including osteoblasts and chondrocytes; these inhibitory effects may be explained by direct targeting of several key regulators that are involved in the G1/S transition of the cell cycle, including the cyclin kinase-dependent kinases (18). In addition, induction of the cell-cycle inhibitor $p21^{WAF1}$ was demonstrated by Cozzolino *et al.* (19) in the hyperplastic parathyroid gland of nephrectomized rats that received 7 d of calcitriol therapy. There is limited information on whether the inhibitory actions of calcitriol on the cell cycle may be amplified in the presence of advanced secondary hyperparathyroidism or whether the accumulated biologically inactive PTH fragments upregulate cell cycle inhibitors; these changes may explain, at least in part, the reported suboptimal response to GH therapy in children with chronic renal failure.

Serum calcitriol levels in uremic rats that received continuous calcitriol infusion increased after 48 h and remained elevated throughout the study period, whereas the serum calcitriol levels increased three to four times the normal range and then declined to baseline levels after 48 h in nephrectomized rats that received boluses of calcitriol (20); these changes in serum calcitriol levels may account for the greater suppression of prepro-PTH synthesis in the parathyroid gland (20). Such increases and decreases in calcitriol levels during intermittent calcitriol therapy may directly affect changes in PTH levels and may explain at least in part the improvement in bone mineral density reported in uremic animals and the enhancement of the molecular markers that reflect chondrocyte maturation and mineralization.

The current study concurs with our previous findings that PTH/PTHrP receptor significantly declined in uremic rats with advanced secondary hyperparathyroidism without any correlation with the circulating serum PTH levels (11). Reduction in chondrocyte proliferation as demonstrated by the decline in PCNA staining and PTH/PTHrP receptor expression may partly account for the impaired growth and shorter bones in animals with renal failure and severe secondary hyperparathy-

roidism. Although studies have suggested a more dominant role of PTH/PTHrP in regulating chondrocyte proliferation, FGF-3 has been reported to play an important role in chondrocyte maturation and vascular invasion (21,22). Deng *et al.* (23) reported that FGFR-3 −/− mice had multiple skeletal abnormalities, including longer and thicker femurs, elongated vertebral body leading to kyphosis, and expanded growth plate cartilage as a result of a widened zone occupied by the hypertrophic chondrocytes. In the current study, there was a considerable decline in the FGFR-3 expression in the Nx-Phos group. In contrast to the histologic findings demonstrated in FGFR-3 −/− mice, the width of the growth plate in the current study was not enlarged and there was no significant increase in bone elongation or tibial length. Our findings were more compatible with the human FGFR-3^{G380R} transgenic mice (hFGFR-3^{G380R}) described by Segev *et al.* (22). Transgenic mice for hFGFR-3^{G380R} had shorter bone growth, smaller chondrocyte columns, irregularly arranged and fewer proliferating and hypertrophic chondrocytes, sparse ossification centers, and the presence of mineralization extensions in the subchondral bone (22). The changes demonstrated in the FGFR-3 expression in the current experiments may be associated with modifications in postreceptor signaling and may suggest delay in chondrocyte maturation and vascular invasion.

BMP-7 or osteogenic protein-1 has an important role in chondrocyte maturation and mineralization. Several studies have reported that BMP-7 promoted mineral formation and terminal differentiation in avian chondrocytes and in mouse fetal bone explants (24,25). In the current study, phosphorus loading in nephrectomized rats diminished BMP-7 protein expression, which may suggest delay in chondrocyte maturation, although our previous experiments have shown that there were no changes in type X collagen expression in these animals. BMP-7 has been used to increase bone formation in adynamic renal bone and eliminate peritrabecular fibrosis in high-turnover bone in animals with renal failure (26,27). Our current experiments have shown that GH alone or with intermittent calcitriol increased BMP-7 protein expression in the phosphorus-loaded rats with advanced secondary hyperparathyroidism; these findings may suggest an increase in chondrocyte maturation and enhancement of mineralization in the chondro-osseous junction. GH therapy has been shown to increase bone mass in children with renal failure (28).

GH is used frequently to augment linear growth in short children with chronic renal failure; however, the severity of the secondary hyperparathyroidism and the concurrent treatment with calcitriol may alter the response to GH therapy. Overall, GH did not improve body length and growth plate width but enhanced selected markers of chondrocyte proliferation and chondrocyte maturation. Overall, the presence of both advanced secondary hyperparathyroidism and concomitant treatment with calcitriol attenuated the anabolic effects of GH in uremic rats. Further studies are required to evaluate whether a higher dose of GH and changes in the frequency of calcitriol administration may overcome the greater degree of GH insensitivity present in phosphorus-loaded rats with renal failure. In addition, it is important to assess whether the higher levels of circulating nonbiologically active PTH fragment may intensify this problem.

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References

1. Wuhl E, Haffner D, Nissel R, Schaefer F, Mehls O: Short dialyzed children respond less to growth hormone than patients prior to dialysis. *Pediatr Nephrol* 10: 294–298, 1996
2. Carter-Su C, Rui L, Herrington J: Role of the tyrosine kinase Jak_2 in signal transduction by growth hormone. *Pediatr Nephrol* 14: 550–557, 2000
3. Gevers EF, Milne J, Robinson ICAF, Loveridge N: Single cell enzyme activity and proliferation in the growth plate: Effects of growth hormone. *J Bone Miner Res* 11: 1103–1111, 1996
4. Kassem M, Blum W, Ristelli J, Mosekilde L, Eriksen EF: Growth hormone stimulates proliferation and differentiation of normal human osteoblast-like cells *in vitro. Calcif Tissue Int* 52: 222–226, 1993
5. Klaus G, Meinhold-Heerlein R, Milde P, Ritz E, Mehls O: Effect of vitamin D on growth cartilage cell proliferation in vitro. *Pediatr Nephrol* 5: 461–466, 1991
6. Mehls O, Knoller N, Oh J, Wesch H, Wunsche B, Schmitt CP: Daily but not pulse calcitriol therapy improves growth in experimental uremia. *Pediatr Nephrol* 14: 658–663, 2000
7. Ardissino G, Schmitt CP, Testa S, Claris-Appiani A, Mehls O: Calcitriol pulse therapy is not more effective than daily calcitriol therapy in controlling secondary hyperparathyroidism in children with chronic renal failure. *Pediatr Nephrol* 14: 664–668, 2000
8. Schmitt CP, Ardissino G, Testa S, Claris-Appiani A, Mehls O: Growth in children with chronic renal failure on intermittent versus daily calcitriol. *Pediatr Nephrol* 18: 440–444, 2003
9. Kuizon BD, Goodman WG, Juppner H, Boechat I, Nelson P, Gales B, Salusky IB: Diminished linear growth during intermittent calcitriol therapy in children undergoing Ccpd. *Kidney Int* 53: 205–211, 1998
10. Goodman WG, Ramirez JA, Belin TR, Choi Y, Gales B, Segre GV, Salusky IB: Development of adynamic bone in patients with secondary hyperparathyroidism after intermittent calcitriol therapy. *Kidney Int* 46: 1160–1166, 1994
11. Sanchez CP, Salusky IB, Kuizon BD, Abdella P, Juppner H, Gales B, Goodman WG: Growth of long bones in renal failure: Roles of hyperparathyroidism, growth hormone and calcitriol. *Kidney Int* 54: 1879–1887, 1998
12. Sanchez CP, He Y-Z: Alterations in the growth plate cartilage of rats with renal failure receiving corticosteroid therapy. *Bone* 30: 692–698, 2002
13. Slatopolsky E, Finch J, Clay P, Martin D, Sicard G, Singer G, Gao P, Cantor T, Dusso A: A novel mechanism for skeletal resistance in uremia. *Kidney Int* 58: 753–761, 2000
14. Schmitt CP, Hessing S, Oh J, Weber L, Ochlich P, Mehls O: Intermittent administration of parathyroid hormone (1-37)

improves growth and bone mineral density in uremic rats. *Kidney Int* 57: 1484–1492, 2000
15. Morales O, Faulds MH, Lindgren UJ and Haldosen L-A: 1α,25-Dihydroxyvitamin D_3 inhibits GH-induced expression of Socs-3 and Cis and prolongs growth hormone signaling via the janus kinase (Jak_2)/signal transducers and activators of transcription ($Stat_5$) system in osteoblast-like cells. *J Biol Chem* 277: 34879–34884, 2002
16. Schaefer F, Chen Y, Tsao T, Nouri P, Rabkin R: Impaired Jak-Stat signal transduction contributes to growth hormone resistance in chronic uremia. *J Clin Invest* 108: 467–475, 2001
17. Klaus G, Weber L, Rodriguez J, Fernandez P, Klein T, Grulich-Henn J, Hugel U, Ritz E, Mehls O: Interaction of Igf-I and 1α,25 $(OH)_2D_3$ on receptor expression and growth stimulation in rat growth plate chondrocytes. *Kidney Int* 53: 1152–1161, 1998
18. Jensen S, Madsen M, Lukas J, Binderup L, Bartek J: Inhibitory effects of 1α,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. *Mol Endocrinol* 15: 1370–1380, 2001
19. Cozzolino M, Lu Y, Finch J, Slatopolsky E, Dusso AS: $P21^{waf1}$ and TGF- mediate parathyroid growth arrest by vitamin D and high calcium. *Kidney Int* 60: 2109–2117, 2001
20. Reichel H, Szabo A, Uhl J, Pesian S, Schmutz A, Schmidt-Gayk H, Ritz E: Intermittent versus continuous administration of 1,25-dihydroxyvitamin D_3 in experimental renal hyperparathyroidism. *Kidney Int* 44: 1259–1265, 1993
21. Amizuka N, Davidson D, Liu H, Valverde-Franco G, Chai S, Maeda T, Ozawa H, Hammond V, Ornitz D, Goltzman D, Henderson J: Signalling by fibroblast growth factor receptor 3 and parathyroid hormone-related peptide coordinate cartilage and bone development. *Bone* 34: 13–25, 2004
22. Segev O, Chumakov I, Nevo Z, Givol D, Madar-Shapiro L, Sheinin Y, Weinreb M, Yayon A: Restrained chondrocyte proliferation and maturation with abnormal growth plate vascularization and ossification in human $Fgfr\text{-}3^{G380r}$ transgenic mice. *Hum Mol Genet* 9: 249–258, 2000
23. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P: Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* 84: 911–921, 1996
24. Nifuji A, Noda M: Coordinated expression of noggin and bone morphogenetic proteins (Bmps) during early skeletogenesis and induction of noggin expression by Bmp-7. *J Bone Miner Res* 14: 2057–2066, 1999
25. Wu LNY, Ishikawa Y, Genge BR, Sampath TK, Wuthier RE: Effect of osteogenic protein-1 on the development and mineralization of primary cultures of avian growth plate chondrocytes: Modulation by retinoic acid. *J Cell Biochem* 67: 498–513, 1997
26. Lund RJ, Davies MR, Brown AJ, Hruska KA: Successful treatment of an adynamic bone disorder with bone morphogenetic protein-7 in a renal ablation model. *J Am Soc Nephrol* 15: 359–369, 2004
27. Gonzalez EA, Lund RJ, Martin KJ, McCartney JE, Tondravi MM, Sampath TK, Hruska KA: Treatment of a murine model of high-turnover renal osteodystrophy by exogenous Bmp-7. *Kidney Int* 61: 1322–1331, 2002
28. Dyck MV, Gyssels A, Proesmans W, Nijs J, Eeckels R: Growth hormone treatment enhances bone mineralisation in children with chronic renal failure. *Eur J Pediatr* 160: 359–363, 2001